Phenotypic Variation Among Three Broiler Pure Lines for Marek's Disease, Coccidiosis, and Antibody Response to Sheep Red Blood Cells[1]

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ABSTRACT To identify candidate genes, chicken lines with the most divergent phenotypes are usually crossed to generate resource mapping populations, for example, either backcrossed or F_2 populations. Linkage between the genetic marker and the phenotypic trait locus is then tested in the mapping population. As an initial step in the development of a mapping population from commercial broilers, the goal of the current research was to evaluate the phenotypic variation among three pure lines for antibody response to SRBC and in resistance to two economically important poultry diseases, Marek's disease (MD) and coccidiosis (*Eimeria acervulina*). Chicks from each line were received and separated into three experimental studies to evaluate each of their responses. In summary, broiler Line 3 had significantly lower antibody responses to SRBC immunizations compared to the other two lines, and nonvaccinated birds from Line 3 were also more susceptible to MD. With coccidiosis, the response was complex, and ranking of the lines was dependent on the age of infection, and whether it was a first or second challenge. With the first challenge, Line 1 was most susceptible at the younger age (Day 30), whereas Line 3 was susceptible at the older age (Day 58). Upon the second challenge, broiler Line 1 remained susceptible at the younger age, but Line 2 was more susceptible at the older age. Line 3 was completely resistant to the second challenge at the older age. Thus, although the broiler lines have been intensively selected for productivity and general livability, this study also demonstrates that the lines differ for immune response and disease resistance. Based on the phenotypic differences between Lines 1 and 3, they were chosen to establish a mapping population for identifying candidate genes that affect MD and coccidiosis in commercial broiler chickens.

(*Key words*: broiler chicken, coccidiosis, Marek's disease, sheep red blood cells, disease resistance)

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INTRODUCTION

Variation in immune responsiveness and disease resistance or susceptibility in chickens has been demonstrated for a variety of antigens and pathogens and for various experimental chicken lines and populations (Gavora et al., 1975; van der Zijpp, 1983; van der Zijpp et al., 1983; Bacon et al., 1984; Dunnington et al., 1989; Bumstead and Barrow, 1993; Parmentier et al., 1994, 1996; reviewed by Lamont, 1998). In many cases, the MHC, or the background genome, or both influence these immune responses and disease associations (Dunnington et al., 1989; Loudovaris et al., 1990a,b; Bacon and Witter, 1993; Yonash et al., 1999a,b). For example, studies have demonstrated that the frequencies of MHC alleles in White Leghorn strains selected for egg production and Marek's disease (MD) resistance differed from that of the nonselected control strain (Gavora et al., 1986; Lakshmanan et al., 1997). In the same Leghorn strains, the association with production traits (high egg production, fertility, hatchability, egg size, and egg quality) and MD resistance also seemed to be dependent on the background genome (Lakshmanan et al., 1997). Similarly, in meat-type chickens, the MHC Class IV region had significant effects in lines that had been selected for high or low early antibody response to *Escherichia coli* vaccination at 10 d of age (Uni et al., 1993). This finding was recently confirmed by Yonash and coworkers (1999b) who showed that the MHC Class I and IV genes,

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Abbreviation Key: MD = Marek's disease; PPI = postprimary immunization.

as well as the *Tap2* genes affected, not only the antibody response to *E. coli*, but also to SRBC and Newcastle disease. The importance of background or non-MHC genes in immune response and disease resistance of chickens has been reported for MD (Vallejo et al., 1997; Bumstead, 1998; Yonash et al., 1999a), *Salmonella* (Hu et al., 1997), coccidiosis (Lillehoj et al., 1989), antibody response to *E. coli* (Yonash et al., 1999b, 2001), and many others.

The commercial broiler chicken has changed dramatically over the decades; growth rate and feed efficiency are parameters that continue to be improved. Broiler chickens are reaching market weights at an earlier age, and it has been estimated that approximately 85 to 90% of this rapid growth is due to genetic selection by the primary breeders (Havenstein et al., 1994). However, these researchers also indicated that the improvements in growth were also associated with higher incidences of leg problems (tibial dyschondroplasia) and general mortality. To reaffirm their findings, Qureshi and Havenstein (1994) compared the immune response of a current commercial broiler strain to that of the Athens-Canadian randombred control strain (developed from early broiler strains in 1957). In their study, they found that the humoral immune response of broiler chickens had declined over the years, whereas cellular functions including macrophages and natural killer cells were not affected. In separate studies, it has also been demonstrated that selection for growth and body weight resulted in reduced immune responsiveness, probably due to negative genetic correlations (van der Zijpp et al., 1983; Parmentier et al., 1996). For example, body weight was negatively correlated to primary antibody response to SRBC (Miller et al., 1992; Parmentier et al., 1996) and resistance to MD (Gavora et al., 1975). In addition to negative genetic correlations, management practices such as vaccination against disease pathogens and isolation of poultry houses have also contributed to the decreased immunoresponsiveness of broilers to pathogens, by reducing the natural selection pressures for genetic resistance. Overall, these data suggest that alternative strategies are needed to improve immune response and disease resistance in broiler chickens, without compromising the gains of current selection practices.

One such strategy is the application of DNA markers in indirect selection for quantitative traits, such as immune response and disease resistance (Cheng, 1997; Dodgson et al., 1997). Over the last few years, DNA markers have been mapped near QTL for growth (Groenen et al., 1997; Van Kaam et al., 1998), feed efficiency (Van Kaam et al., 1999a), carcass traits (Van Kaam et al., 1999b), resistance to Marek's disease (Yonash et al., 1999a), and antibody response and survival to *E. coli* (Yonash et al., 1999b, 2001). All of these QTL mapping studies were conducted in experimental chicken populations; few studies have been initiated in commercial populations mainly due to the outbred nature of the lines. In addition, genetic loci within the commercial lines are subject to fixation due to intensive selection, and, therefore, specialized crosses are also needed to generate a mapping population that is segregating for the genes of interest. The commercial broiler chick is produced from a cross of three pure lines that are selected for different growth and reproductive traits but not immune response or specific disease resistance. Therefore, as an initial step in QTL mapping of genes affecting immune response and disease resistance in broiler chickens, the goal of this study was to characterize the response of the pure lines to a natural antigen, SRBC, as well as to two economically important poultry diseases, MD and coccidiosis. Based on the results of this study, the two broiler pure lines that are most phenotypically distinct, and assumed to be genetically different, will be mated in a line cross to generate an F_2 mapping population that is segregating for genes affecting immune response and disease resistance.

MATERIALS AND METHODS

Experimental Lines

Three broiler chicken pure lines that are used in crosses to produce the commercial broiler or roaster chicks were evaluated in this study, and they were randomly designated Lines 1, 2, and 3, respectively. Chicks from each line were hatched at the broiler breeding company and then transported to the University of Delaware or USDA-ARS (Beltsville, MD) for immune response and disease challenge studies. A random group of chicks from each broiler line was evaluated in separate studies for antibody response to SRBC and challenges for Marek's disease and coccidiosis. Chicks were provided water and feed ad libitum during all experiments.

Antibody Response to SRBC

Male chicks were intermingled in two floor pens (n = 17 or 18 chicks/line per pen; total of 35 chicks/line) and were tested for their antibody response to SRBC at 3 wk of age. A pre-immune blood sample (Day 0) was collected from all chicks, and then each chick was injected intramuscularly at two sites in the breast with 0.5 mL/site of a 25% SRBC suspension in PBS (van der Zijpp et al., 1983). Blood samples were collected Day 5 postprimary immunization (PPI). Chicks received a booster or second immunization on Day 7 PPI, with blood collection on Day 12 PPI. All blood samples (2 to 3 mL) were allowed to clot overnight at 4 C, and sera were harvested by centrifugation, heat-inactivated at 56 C for 30 min, and then stored at −20 C until assayed. Total anti-SRBC hemagglutinating antibodies were measured in 96-well, U-bottomed microtiter plates. Briefly, an equal volume of 0.5% SRBC suspension in PBS was added to each serum dilution (final volume of 50 μL), samples were mixed, and then they were allowed to react at room temperature for 2 h. The hemagglutination titer for a serum was the reciprocal, expressed as log2, of the highest serum dilution that gave 100% agglutination.

Marek's Disease Challenge

Nonvaccinated male chicks (n = 50 per line) were housed in four small colony units at 1 d of age. Each

chick received 400 plaque-forming units of a Marek's disease virus strain, RB1B, intraabdominally at 5 d of age. The RB1B strain has been described previously (Schat et al., 1982) and was in its ninth passage in chick embryo fibroblasts for this challenge. Chicks were observed for clinical signs and mortality to 8 wk postchallenge. All birds that died during the challenge were necropsied and examined for gross lesions (MD tumors). At the end of the 8-wk challenge, remaining birds were euthanized and examined for gross MD lesions.

Coccidiosis Challenge

One-day-old male broiler chicks (n = 36 per line) were maintained in battery brooder cages until challenged. Chicks from each line were separated into three treatment groups based on the age of challenge. First infections were given at 30, 37, and 58 d of age; 12 chicks from each line for each age group were orally challenged with 10,000 sporulated oocysts of a field strain of *Eimeria acervulina* that is maintained at the Parasite Biology, Epidemiology, Systematics Laboratory, USDA-ARS. Second challenges of the three groups were performed 2 wk after the first challenge (Days 44, 51, and 72, respectively). During first and second infections, oocyst production was measured by collecting fecal samples of individual birds from 5 to 9 d postinfection. Fecal samples were homogenized in water and oocysts were enumerated as described previously (Lillehoj et al., 1988).

Statistical Analyses

Total anti-SRBC serum antibody titers (transformed to $\log_2$) and oocyst numbers during the coccidiosis challenge were analyzed by general linear models ANOVA (SAS Institute, 1996). In the ANOVA, pen, (pen × line) and (pen × time of serum collection) were not significant, and, therefore, data were pooled for antibody response to SRBC. Broiler line, time of serum collection (Day 5 PPI or Day 12 PPI), and their interaction were main effects in the anti-SRBC antibody analysis. Broiler line, age of infection, challenge period (first vs. second) and their interactions were main effects for the coccidiosis data. Multiple mean comparisons for the SRBC and coccidiosis studies were tested with Duncan's multiple-range test. For the MD challenge data, birds were categorized as MD positive (presence of paralysis or tumors) or MD negative (absence of paralysis and tumors). Total MD incidence was then calculated as a percentage of challenged chickens, and the data were analyzed using a chi-squared test with a 2 × 3 contigency table. The null hypothesis tested the independence of broiler line and MD incidence. Data for percentage MD mortality were analyzed in a similar manner. For statistical analyses, significance was considered at $P < 0.05$.

RESULTS

Pre-immune sera (Day 0) from all birds were negative against SRBC in the hemagglutination assay. The effect

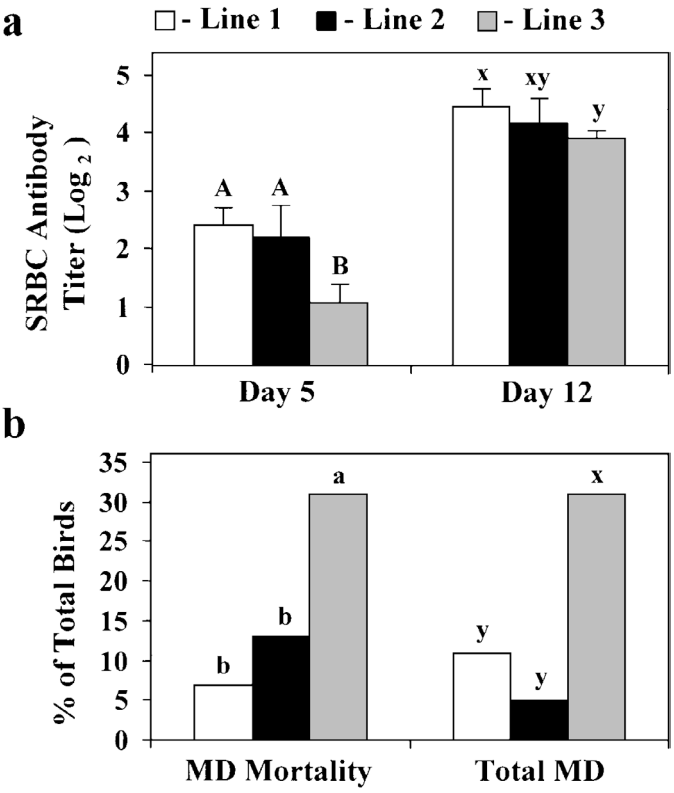

FIGURE 1. Phenotypic variation in antibody response to SRBC (a) and Marek's disease (MD) resistance (b) among three broiler pure lines. The total anti-SRBC antibody titers with standard errors, after first (Day 5 postprimary infection) and second (Day 12 postprimary infection) immunizations, were determined in a group of male chicks (n = 35 per line). A second group of nonvaccinated male chicks (n = 50 per line) was challenged with very virulent Marek's disease virus, RB1B, and MD mortality and total MD (including paralysis, tumors, and mortality) were determined as a percentage of total birds. Different letters within a group indicate statistically significant differences among the broiler lines ($P < 0.05$).

of line and time of serum collection were statistically significant for antibody response to SRBC by ANOVA. The responses of the three broiler lines to SRBC after first and second immunizations are shown in Figure 1a. Line 3 birds had significantly lower antibody responses to SRBC after the first immunization (Day 5 PPI) than birds from Lines 1 and 2. With a second immunization (Day 12 PPI), birds of Line 3 had anti-SRBC antibody titers that were significantly lower than those of Line 1 ($P = 0.0301$). Lines 1 and 2 had similar total anti-SRBC antibody titers after the first and second immunizations. The greatest difference in antibody titers against SRBC among the broiler lines were after the first immunization. Marek's disease mortality and total MD incidence were significantly different among the three broiler lines (Figure 1b). Percentage MD mortality and total MD incidence were significantly higher in Line 3, whereas the MD traits between Lines 1 and 2 were comparable and not significant.

For coccidiosis, broiler line, age of infection, challenge period (first vs. second) and their interactions were significant by ANOVA. After the first infection, the number of oocysts shed varied among the broiler lines; however,

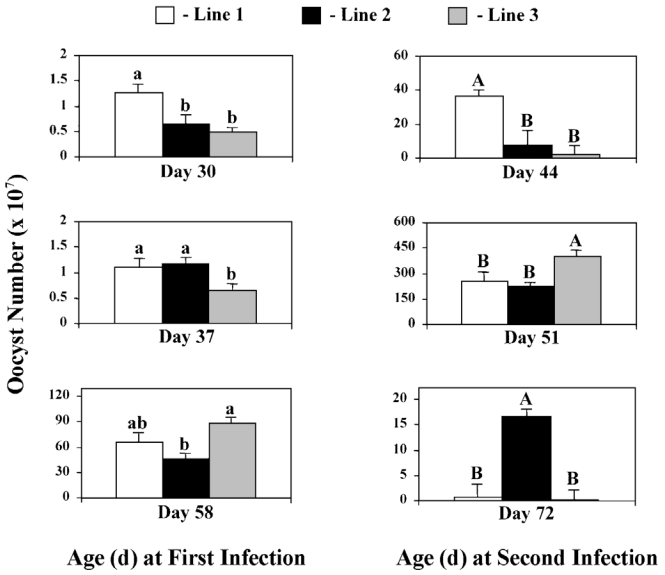

FIGURE 2. Phenotypic variation in response to first and second *Eimeria acervulina* challenges among three broiler pure lines. Male chicks (n = 12 per line per challenge age) were orally challenged with *E. acervulina* oocysts at three different ages (Days 30, 37, and 58). Second challenges were 2 wk later (Days 44, 51, and 72). Susceptibility to the coccidial infection was determined by oocyst counts in excreted feces. Total oocyst numbers with standard deviations are given, and different letters within a group indicate statistically significant differences among the broiler lines ($P < 0.05$).

the line variation was dependent on the age of infection (Figure 2). At the early age (Day 30), oocyst production after primary inoculation with *Eimeria acervulina* was greatest in Line 1 (more susceptible) and lowest in Line 3 (more resistant). In contrast, at the older age (Day 58), Line 3 was more susceptible and it shed significantly more oocysts than Line 2. Oocyst shedding from Line 1 birds was not significantly different from Line 3 birds at this time point. At the intermediate age, birds from Lines 1 and 2 shed significantly more oocysts than birds from Line 3. With a secondary challenge of *E. acervulina*, Line 1 birds continued to be highly susceptible after the early age of infection (Day 44), whereas at an older age (Day 72), Line 2 birds shed significantly higher oocysts than birds from Lines 1 and 3. The Line 3 birds were generally more resistant at these time points; however, at the intermediate age (Day 51), the Line 3 birds shed higher oocyst numbers than birds from Lines 1 and 2. Mortality was highest in the older birds challenged at Day 72 with one, three, and four birds dying in Lines 1, 2, and 3, respectively.

DISCUSSION

Although the commercial broiler lines in this study have been under intensive selection for high productivity and general livability, phenotypic variations in immune response and disease resistance among the lines were apparent. Line 3 birds demonstrated the poorest performance in antibody response to SRBC and to challenge with MD. The other two broiler lines were comparable in their responses to these agents. It was not unexpected that Line 3 had low antibody titers to SRBC as well as an increased susceptibility to MD. Previous reports have demonstrated that chickens selected for high antibody response to SRBC also had enhanced immune responses to other T-dependent antigens (BSA, *E. coli*) and to vaccination with Newcastle disease virus or other avian pathogens (Gross et al., 1980; Parmentier et al., 1994, 1996). Conversely, meat-type chickens selected for high antibody response to *E. coli* also responded well to SRBC, BSA, and Newcastle disease virus (Heller et al., 1992). Therefore, our data are consistent with these findings and suggest that Line 3 birds had an overall reduced immune response compared to birds from Lines 1 and 2.

Variation in antibody response to SRBC among different chicken lines or populations has been reported previously (van der Zijpp, 1983; Martin et al., 1989; Loudovaris et al., 1990a). In one study, total antibody levels to SRBC after primary immunization were influenced by the genetic line, and the differences in antibody levels were due to the rate of production and persistence of the antibodies, in particular IgG (Martin et al., 1989). With the first immunization of SRBC, the majority of the antibodies were IgM on Day 5 PPI, regardless of the genetic line. With the second immunization (Day 24 PPI), the line that was selected for high antibody response to SRBC produced mainly IgG on the fifth day postsecondary immunization, whereas the line that was selected for low antibody response to SRBC produced mainly IgM. However, it should be noted that IgM and IgG were detected after the second immunization in both genetic lines. Although the IgM and IgG levels were not determined in this study, it is speculated that the differences in antibody response to SRBC among the broiler lines probably reflect differences in the rate of production or persistence of the IgM and IgG isotypes. Genetic control of antibody response to SRBC in chickens is largely due to genes within the MHC (Dunnington et al., 1989; Loudovaris et al., 1990a). In addition, Dunnington and coworkers (1989) reported that the background genome of White Leghorns also influenced the primary antibody response to SRBC. Similarly, differences among chicken lines and breeds for MD resistance and susceptibility have been reported by several groups, and MHC and non-MHC genes are involved (Hansen et al., 1967; Gavora et al., 1975; Longnecker et al., 1976; Briles et al., 1982; Vallejo et al., 1997; Yonash et al., 1999a). High rates of egg production in Leghorn strains and low growth rates in meat-type chickens are associated with MD resistance (Gavora et al.,1975). Our data are consistent with these findings; the Line 3 birds tended to be faster growing and larger than birds in Lines 1 and 2; they were also more highly susceptible to MD.

The severity of a coccidiosis challenge at different ages among the three broiler lines was determined by measuring oocyst production after the first and second infections with *Eimeria acervulina*. Similar to previous

findings, older birds were more susceptible to coccidial infection, as demonstrated by higher mortality and increased numbers of oocysts shed (Long, 1968). Our findings are in contrast to Gross and coworkers (1980) who demonstrated that chickens selected for high antibody response 5 d postintravenous injection of SRBC were more resistant to a challenge with *Eimeria necatrix* than chickens selected for low antibody response to SRBC. Although broiler Line 1 had the highest antibody titer to SRBC, it was the most susceptible to coccidiosis, especially in young birds (Day 30). Similarly, broiler Line 3 had a low antibody titer to SRBC but was more resistant to coccidiosis at the same age of infection. The differences between these two studies may be explained by the complex coccidial response that seems to be dependent on the age of infection of the birds and the type of challenge, i.e., primary vs. secondary, as demonstrated in our study. When age of infection is taken into account (Day 58 is comparable for both studies), our data are in agreement with Gross and coworkers (1980). The coccidiosis data are also consistent with previous findings that suggest two separate mechanisms for determining resistance to coccidiosis (Lillehoj, 1991; Lillehoj et al., 1999). An innate mechanism after primary infection and acquired immunity after secondary infection are involved in protective immunity to coccidiosis. It may be that the innate mechanisms are at higher levels or they have maturated to adult levels more quickly in birds from Lines 2 and 3 compared to Line 1 birds. Therefore, Lines 2 and 3 birds are more resistant to coccidiosis at earlier ages than Line 1 birds. The efficiency of the innate mechanisms and their role in antigen presentation to T lymphocytes also resulted in enhanced acquired immunity in these lines with secondary challenge. In contrast, in older birds, in which innate and acquired immune responses have matured to adult levels, the Line 3 birds were the most susceptible to coccidiosis, suggesting that they have an overall poorer natural immunity to the *Eimeria* parasite. However, upon secondary challenge, acquired immunity in the Line 3 birds was very efficient and the birds were resistant. This observation is in contrast to the Line 2 birds that were more susceptible at older ages upon secondary challenge. Therefore, the data from this study demonstrate the complexity of immune responses to coccidiosis. At the genetic level, both MHC and non-MHC genes have been implicated in the control of these host immune responses to coccidiosis (Johnson and Edgar, 1986; Ruff and Bacon, 1989; Lillehoj et al., 1989). Interestingly, Johnson and Edgar (1982) divergently selected for natural resistance and susceptibility to *Eimeria tenella* and they found that selection was rapid, indicating that only a few genes are involved.

Genetic resistance to disease is more critical in broiler chickens due to the shortened time to market and the time required to develop protective immunity with vaccination. Based on our findings in this study, genes that influence natural immunity or stimulate an early acquired immunity to MD or coccidiosis should be considered as candidates in selection programs for disease resistance in broilers. The obvious benefits are clearly demonstrated in birds that were selected for early and high antibody response to *E. coli* (Heller et al., 1992). The current report demonstrates that there are phenotypic variations in antibody response to SRBC, as well as response to two economically important diseases, MD and coccidiosis, among the broiler pure lines. Although the data imply that the differences may be due to genetic variation among the lines, the role of maternal effects must also be considered. However, based on the moderate heritabilities for immune response in chickens (van der Zijpp et al., 1983; Kaiser et al., 1997, 1998), it is anticipated that the phenotypic differences in these broiler lines are partially genetic in nature. Broiler Lines 1 and 3 were the most phenotypically distinct, based on antibody response to SRBC, resistance to MD, and early resistance to *Eimeria* challenge. Therefore, these two broiler lines will be crossed to develop F_2 mapping populations that are segregating for genes affecting resistance to MD and coccidiosis. The mapping populations will then serve as the basis for characterizing MHC and non-MHC genes and their roles in resistance to MD and coccidiosis.

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